

A Yeast Estrogen Screen for Examining the Relative Exposure of Cells to Natural and Xenoestrogens

Steven F. Arnold,^{1,2} Matthew K. Robinson,³ Angelo C. Notides,³ Louis J. Guillette, Jr.,^{1,4} and John A. McLachlan^{1,2,5}

¹Tulane-Xavier Center for Bioenvironmental Research, New Orleans, LA 70112 USA; ²Department of Environmental Health Sciences, Tulane University School of Public Health and Tropical Medicine, New Orleans, LA 70112 USA; ³Department of Biochemistry, University of Rochester Medical Center, Rochester, NY 14642 USA; ⁴Department of Zoology, University of Florida, Gainesville, FL 32611 USA; ⁵Department of Pharmacology, Tulane University Medical School, New Orleans, LA 70112 USA

Xenoestrogens, such as *o,p'*-DDT and octyl phenol (OP), have been associated with reproductive abnormalities in various wildlife species. Xenoestrogens mimic the natural estrogen 17 β -estradiol and compete for binding to the estrogen receptor. Even though the affinity of *o,p'*-DDT and OP for the estrogen receptor is approximately 1000-fold lower than 17 β -estradiol, the actions of xenoestrogens could be enhanced if their bioavailability in serum were greater than 17 β -estradiol. To test this hypothesis, the yeast estrogen screen (YES) was created by expressing human estrogen receptor (hER) and two estrogen response elements (ERE) linked to the *lacZ* gene. The β -galactosidase activity of the YES system was significantly increased after treatment with 17 β -estradiol or the xenoestrogens diethylstilbestrol (DES), *o,p'*-DDT, and OP but not with vehicle, antiestrogen ICI 164,384, dexamethasone, or testosterone. To determine whether serum proteins affected the bioavailability of natural estrogens compared to xenoestrogens, albumin, sex hormone binding globulin (SHBG), or charcoal-stripped serum were added to the YES system and β -galactosidase activity assayed. Albumin and SHBG decreased β -galactosidase activity in the presence of estradiol to a greater extent than DES, *o,p'*-DDT, and OP. Human and alligator charcoal-stripped serum were also effective at selectively reducing β -galactosidase activity in the presence of estradiol compared to xenoestrogens. Human serum was more effective than alligator serum in reducing β -galactosidase activity in the presence of xenoestrogens, indicating that serum may serve as a biomarker for sensitivity to xenoestrogens. Selective binding of 17 β -estradiol by proteins in serum indicates that certain xenoestrogens may exert greater estrogenicity than originally predicted. The estrogenic potency of a compound involves its binding affinity, bioavailability in serum, and persistence in the environment. Our data demonstrate the utility of the YES system for identifying and characterizing environmental estrogens. **Key words:** albumin, alligator, estrogens, sex hormone binding globulin, xenoestrogens, yeast estrogen screen. *Environ Health Perspect* 104:544–548 (1996)

It has been hypothesized that decreased fertility and increased sexual dysfunction in wildlife are associated with endocrine-related toxicants (1,2). For example, fish exposed to various endocrine-mimicking compounds (e.g., TCDD, polychlorinated biphenyls, or *o,p'*-DDT) exhibit smaller gonads, reduced egg output, abnormal plasma sex hormone concentrations, and greater embryonic mortality (3,4). Various xenoestrogens, such as *o,p'*-DDT (5) and octyl phenol (OP) (6) that compete with the natural estrogen 17 β -estradiol for binding to the estrogen receptor have been identified. The estrogen receptor is a member of a superfamily of transcription factors that transduce their intracellular signals by binding lipophilic hormones. Natural estrogens play a major role in vertebrate reproduction. Estrogens are also associated with an increased risk for cancers of the breast, ovary, and endometrium in human populations (7,8). It has been hypothesized that the effects observed in wildlife exposed to xenoestrogens could serve as biomarkers for public health (1,2,9).

Endocrine signaling is a complex process involving synthesis, release, uptake, and the eventual degradation of hormones. The physiological response generated in the target cell due to a hormone is a composite of these processes. Thus, the effective concentration is that concentration capable of inducing a specific response in the target cell and, in the case of estradiol, is modified by binding to serum proteins such as albumin and sex hormone binding globulin (SHBG). Albumin is a nonspecific binding protein with a low affinity and specificity for estradiol, whereas SHBG has a high affinity and specificity for estradiol. The synthetic estrogen diethylstilbestrol (DES) has been shown to have a much lower affinity for SHBG than estradiol. Therefore, at equivalent concentrations of estradiol and DES, the concentration of DES at the target cell will be significantly greater than that of estradiol.

Recent studies examining a number of wildlife species have demonstrated that exposure to contaminants can permanently modify the embryonic development of the

reproductive and endocrine systems (2). One well-documented case is that of the alligators living in Lake Apopka, Florida. The alligators were exposed to a number of anthropogenic contaminants derived from agricultural and municipal activity as well as a major pesticide (dicofol and DDT) spill (10–12). Alligator eggs from Lake Apopka show elevated levels of *p,p'*-DDE, *p,p'*-DDD, dieldrin, and various polychlorinated biphenyls (PCBs) (13). Hatchlings from these eggs exhibited abnormal gonadal anatomy, gonadal steroidogenesis, and plasma sex steroid concentrations (11,12). Recent data suggest that the abnormalities in plasma sex-steroid concentrations are partly due to modifications in the serum binding capabilities for the various sex steroids (12). Variations in serum binding will modify circulating sex steroid levels and will also modify the transport and cellular availability of various xenoestrogens. Although serum binds naturally occurring sex steroids, it does not appear that serum proteins interact, to any great extent, with synthetic estrogens, such as DES (14) or with environmental estrogens such as *o,p'*-DDT (15).

In order to measure the estrogenicity of a certain compound, several factors must be taken into account: 1) affinity of the compound for the estrogen receptor, 2) accumulation of the compound in the environment and the body, 3) degradation or metabolism of the compound in the envi-

Address correspondence to S. F. Arnold, Tulane-Xavier Center for Bioenvironmental Research, New Orleans, LA 70112 USA.

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ronment and body, and 4) the availability of the compound to the target cell. The E-screen assay has been developed to identify environmental compounds that demonstrate estrogenicity (16,17). This assay uses the proliferation of MCF7 breast carcinoma cells as a marker of estrogenicity. We created a simpler approach, the yeast estrogen screen (YES), using a yeast strain responsive to estrogens. Yeast do not contain sex steroid or thyroid hormone receptors, except those transfected into the strain, but they possess proteins homologous to mammalian cells that are required for activated transcription. In this paper, we present the use of the YES system for the screening of xenoestrogens. In addition, the YES system was used to assess the relative bioavailability of estradiol compared to the synthetic estrogens DES, *o,p'*-DDT, and OP.

Materials and Methods

We purchased 17 β -estradiol, DES, *o,p'*-DDT [1,1,1-trichloro-2-(*p*-chlorophenyl)-2-(*o*-chlorophenyl)ethane], 4-OP, human albumin, and human SHBG from Sigma Chemical Co. (St. Louis, Missouri). The yeast reporter plasmid YRPE2, containing 2 EREs linked to the *lacZ* gene, was a gift from B. O'Malley and Z. Nawaz, Baylor Medical College.

The yeast expression plasmid, pSCW231-hER, was created by ligating an *Eco*R1 fragment of the cDNA of the hER into pSCW231. The yeast strain BJ2407 was transformed with pSCW231 or pSCW231-hER and YRPE2 using methodology described by Chen et al. (18). Transformants were selected for 3 days at 30°C on plates by tryptophan and uracil auxotrophy.

A single yeast colony was grown in SD-uracil, tryptophan medium overnight at 30°C. The next day, 50 ml of the overnight culture was diluted into 200 ml of fresh medium and grown overnight in the presence of the test compounds. All compounds were prepared in dimethylsulfoxide (DMSO) and added such that the concentration of DMSO did not exceed 2%. Human albumin and SHBG were dissolved in SD-uracil, tryptophan and added at the appropriate concentration to the culture. Alligator (*Alligator mississippiensis*) serum was obtained from a gravid, adult female collected under permit from the Florida Game and Fresh Water Fish Commission. Samples were taken from the postcranial sinus and allowed to clot for 2 hr at room temperature. The serum tubes were centrifuged and sera were collected, snap frozen in liquid nitrogen, and stored at -70°C. The human serum was taken from two adult men and two adult women.

Samples were collected from the brachial blood vessels and treated as described above. Before use in the YES system, serum was charcoal-stripped by incubation with 5% Norit-A overnight at 4°C. The serum was separated from the charcoal by centrifugation and the protein concentration determined using the Bio-Rad (Hercules, California) protein assay. The protein concentration of the alligator serum was 70 mg/ml and of the human serum was 55 mg/ml. The inclusion of albumin, SHBG and sera from human and alligator at the concentrations used did not significantly affect the growth of the yeast.

For the β -galactosidase assays, the yeast cells were collected by centrifugation and resuspended in 700 μ l of Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 35 mM β -mercaptoethanol). The cells were permeabilized by the addition of 6 ml of CHCl₃ and 4 ml of 0.1% sodium dodecyl sulfate followed by vortexing for 25 sec. The reactions were equilibrated at 30°C for 10 min, then 160 μ l ONGP (4 mg *o*-nitrophenyl β -D-galactopyranoside/ml Z-buffer) was added and the reactions returned to 30°C. The reactions were terminated by the addition of 400 μ l 1M NaCO₃, the cell debris was removed by centrifugation at 15,000g for 5 min, and the absorbance (A) at 420 nm was measured. Miller units were determined using the following formula: $[A_{420}/(A_{600} \text{ of } 1/10 \text{ dilution of cells} \times \text{volume of culture} \times \text{length of incubation})] \times 1000$.

Results

Identification of Xenoestrogens by the YES System

The yeast strains SCW-ERE and hER-ERE were grown overnight in the presence or absence of 17 β -estradiol, DES, *o,p'*-DDT, OP, antiestrogen ICI 164,384, dexamethasone, or testosterone. The yeast strain SCW-ERE did not exhibit increased β -galactosidase activity when incubated with the various compounds listed above (data not shown). This was expected because yeast do not contain a naturally responsive estrogen receptor. In contrast, the β -galactosidase activity of yeast strain hER-ERE was significantly increased after treatment with estradiol, DES, *o,p'*-DDT, or OP, but not with ICI 164,384, dexamethasone, or testosterone (Fig. 1). These results indicate that the β -galactosidase activity of yeast strain hER-ERE was selectively increased after treatment with natural estrogens or xenoestrogens. A protein extract was prepared after strain hER-ERE was incubated in the presence or absence of estradiol. The

extract was separated by sodium dodecyl sulfate-gel electrophoresis and transferred to a polyvinylidene difluoride membrane for Western blot analysis. The extract contained similar levels of hER in the presence and absence of estradiol or xenoestrogens (data not shown).

To more precisely define the potency of the various estrogens in strain hER-ERE, a dose-response experiment, with various concentrations of the estrogens, was performed. The EC₅₀ (the concentration required for 50% transcription) of estradiol and DES was approximately 0.2 nM (Fig. 2). The K_d of hER for estradiol in yeast was

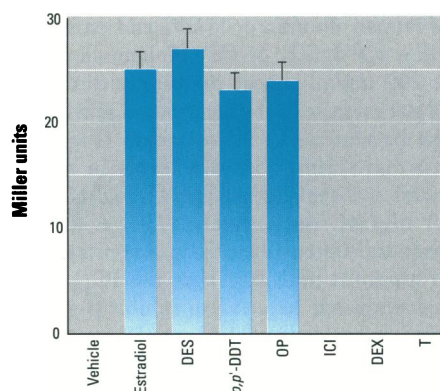


Figure 1. β -Galactosidase activity of yeast strain hER-ERE after treatment with various compounds. Strain hER-ERE was grown overnight in the presence of various compounds at a concentration of 10 μ M. The activity of β -galactosidase was measured the next morning and was calculated as Miller units as described in Materials and Methods. Each value represents the mean of three independent experiments with three replicates. DES, diethylstilbestrol; OP, octyl phenol; DEX, dexamethasone; T, testosterone.

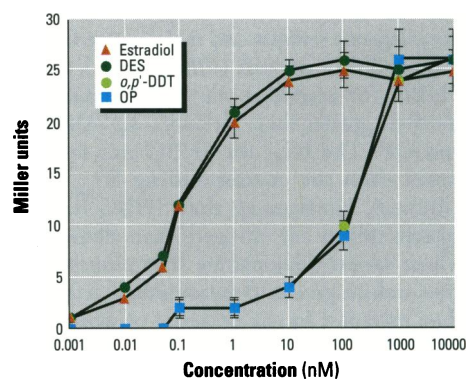


Figure 2. A dose-response curve of yeast strain hER-ERE after treatment with increasing concentrations of 17 β -estradiol, diethylstilbestrol (DES), *o,p'*-DDT or octyl phenol (OP). Strain hER-ERE was grown overnight in the presence of the indicated estrogens at increasing concentrations. The activity of β -galactosidase was measured the next morning and was calculated as Miller units as described in Materials and Methods. Each value is the mean of three independent experiments with three replicates.

0.2 nM, indicating that the EC_{50} measured in yeast is within physiological range (unpublished data). The EC_{50} for *o,p'*-DDT and OP was 200 nM, or 1,000-fold greater than estradiol and DES. These results are also consistent with the affinity of *o,p'*-DDT and OP for the estrogen receptor, calculated at 1,000-fold less than estradiol.

Bioavailability of Estrogens in the YES System with Human Albumin and SHBG

To evaluate the bioavailability of natural estrogens compared to xenoestrogens, yeast strain hER-ERE was grown in the presence of human albumin or SHBG and estradiol, DES, *o,p'*-DDT, or OP. Human albumin at 200 mg/ml almost completely eliminated the increase in β -galactosidase activity in the presence of 17 β -estradiol (Fig. 3). Normal serum albumin levels in adult males and females average 45 mg/ml and 41 mg/ml, respectively (19). A dose-response curve with various concentrations of albumin indicated that the IC_{50} (the concentration required to inhibit β -galactosidase activity by 50%) was 12.5 mg/ml. Albumin was a less effective inhibitor of β -galactosidase activity in the presence of DES, *o,p'*-DDT, and OP, with an IC_{50} of approximately 100 mg/ml. Albumin was 1.7-fold more effective in reducing β -galactosidase activity in the presence of estradiol compared to DES, *o,p'*-DDT, and OP. SHBG was significantly more effective than albumin in reducing β -galactosidase activity. The IC_{50} for SHBG was 0.035 mg/ml for estradiol, 0.15 mg/ml for DES, *o,p'*-DDT, and 0.1 mg/ml for OP (Fig. 4). Normal serum concentrations of SHBG in nonpregnant females and males range from 0.003–0.015 mg/ml (20). The IC_{50} of SHBG for estradiol was approximately fourfold greater than for DES, *o,p'*-DDT, and OP. The IC_{50} for SHBG was much lower than the corresponding IC_{50} for albumin, confirming that SHBG has a greater affinity for estrogens than albumin. These results demonstrate that xenoestrogens induce greater β -galactosidase activity than estradiol in the presence of albumin or SHBG, suggesting that the availability of xenoestrogens is greater than estradiol in the presence of albumin or SHBG.

Bioavailability of Estrogens in the YES System with Serum

We evaluated the availability of natural estrogens and xenoestrogens in the presence of whole human or alligator serum. The yeast strain hER-ERE was grown overnight in the presence of human or alligator charcoal-stripped serum containing

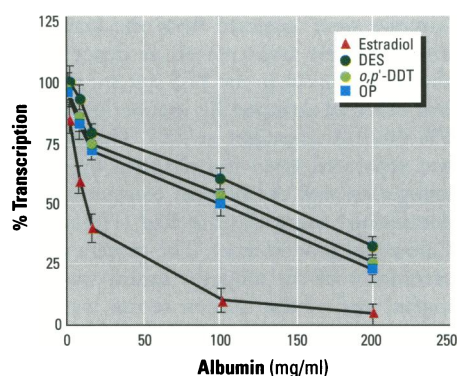


Figure 3. β -Galactosidase activity of yeast strain hER-ERE after treatment with albumin and natural or xenoestrogens. Strain hER-ERE was grown overnight in the presence of increasing concentrations of albumin and 17 β -estradiol (10 nM), diethylstilbestrol (DES; 10 nM), *o,p'*-DDT (10 μ M), or octyl phenol (OP; 10 μ M). The concentrations of the xenoestrogens used in this assay were the lowest concentration needed to induce 100% β -galactosidase activity in Figure 2. Concentrations of albumin greater than 200 mg/ml were not used because they significantly inhibited yeast growth. β -galactosidase activity was measured as described in Materials and Methods and is expressed as a percentage of 100% transcription in the absence of albumin. Each value is the mean of three independent experiments with three replicates.

17 β -estradiol, DES, *o,p'*-DDT, or OP. β -Galactosidase activity induced by 17 β -estradiol was selectively inhibited with human serum compared to DES, *o,p'*-DDT, and OP (Fig. 5A). The IC_{50} for human serum was 1.5 mg protein/ml for 17 β -estradiol and 7 mg protein/ml for DES, *o,p'*-DDT, and OP. The IC_{50} for alligator serum was 2 mg protein/ml for 17 β -estradiol, similar to the IC_{50} obtained with human serum (Fig. 5B). The concentrations of alligator serum used decreased β -galactosidase activity by only 30–40% (Fig. 5B). Concentrations of serum greater than 10 mg protein/ml were not used because they substantially inhibited the growth of the yeast (data not shown). These results indicate that human and alligator serum selectively decrease the β -galactosidase induced by 17 β -estradiol compared to xenoestrogens. In addition, the alligator serum appears to contain less binding activity than human serum for xenoestrogens, but not estradiol.

Discussion

We developed the YES system to identify and characterize compounds that act as estrogens through the binding of the hER. The simplicity of this screen will allow not only the identification of estrogens, but also mechanistic studies with estrogens. The YES system responded to the known estrogens, 17 β -estradiol, DES, *o,p'*-DDT,

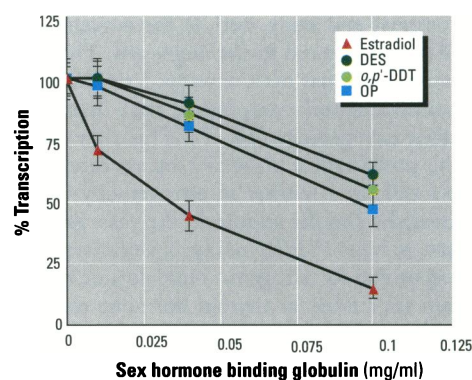


Figure 4. β -Galactosidase activity of yeast strain hER-ERE after treatment with sex hormone binding globulin (SHBG) and natural or xenoestrogens. Strain hER-ERE was grown overnight in the presence of increasing concentrations of SHBG and 17 β -estradiol (10 nM), diethylstilbestrol (DES; 10 nM), *o,p'*-DDT (10 μ M), or octyl phenol (OP; 10 μ M). The concentrations of the xenoestrogens used in this assay were the lowest concentration needed to induce 100% β -galactosidase activity in Figure 2. β -galactosidase activity was measured as described in Materials and Methods. Concentrations of SHBG > 0.1 mg/ml were not used because they significantly inhibited yeast growth. β -galactosidase activity is expressed as a percentage of 100% transcription in the absence of SHBG. Each value is the mean of three independent experiments with three replicates.

and OP, but not the antiestrogen ICI 164,384, dexamethasone, or testosterone. A dose-response experiment with estradiol, DES, *o,p'*-DDT, or OP indicated that the EC_{50} values measured in yeast for the various estrogens were consistent with their affinity for the estrogen receptor. Interestingly, the EC_{50} for *o,p'*-DDT and OP is 200 nM, well within the reported population exposure of these compounds (6,21,22). Our results and reports from other laboratories indicate that the activation of estrogen receptors by estrogen is similar in yeast and mammalian cells (23; Arnold and McLachlan, unpublished data). There are several advantages of the YES system compared to mammalian cells: 1) yeast are inexpensive to maintain, 2) the expression and reporter plasmids in the yeast strain are maintained by growth in selective media, and 3) the β -galactosidase assay is straightforward and allows for large-scale screening of compounds. The potential disadvantage of the YES system is that it is unclear whether yeast will metabolize proestrogens to estrogens. Therefore, the results in the YES system will require confirmation in mammalian cells.

The inclusion of albumin or SHBG in the YES system selectively decreased β -galactosidase activity induced by estradiol compared to the xenoestrogens, DES, *o,p'*-DDT and OP. The same results were

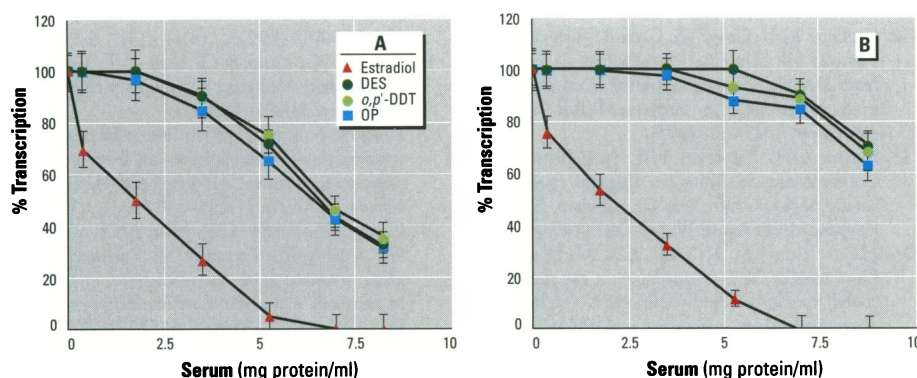


Figure 5. β -Galactosidase activity of yeast strain hER-ERE after treatment with (A) human or (B) alligator serum and natural estrogens or xenoestrogens. Strain hER-ERE was grown overnight in the presence of increasing concentrations of human or alligator whole charcoal-stripped serum and 17 β -estradiol (10 nM), diethylstilbestrol (DES; 10 nM), *o,p'*-DDT (10 mM), or octyl phenol (OP; 10 mM). The concentrations of the xenoestrogens used in this assay were the lowest concentration needed to induce 100% β -galactosidase activity in Figure 2. Concentrations of serum >7.5 mg protein/ml were not used because they significantly inhibited yeast growth. β -Galactosidase activity was measured as described in Materials and Methods and is expressed as a percentage of 100% transcription in the absence of serum. Each value is the mean of three independent experiments with three replicates.

obtained using human and alligator sera. Our results are consistent with those observed by vom Saal et al. (15), who used MCF7 cells to assess the bioavailability of estradiol, DES, *o,p'*-DDT, and methoxychlor. They reported that DES and *o,p'*-DDT were more available in serum compared to serum-free media than estradiol and methoxychlor, thus stimulating greater cell proliferation. Together, these results begin to provide the framework needed to determine the basis by which the estrogenicity of compounds is calculated. It suggests that numerous factors are involved. First, the binding affinity is important for determining the interaction of the ligand, in this case estrogens, with their receptor at the molecular level. Second, the availability of the ligand in serum will influence the concentration at the target cell. The concentration at the target cell will determine the degree of responsiveness produced by that cell. *o,p'*-DDT and OP are classified as weak estrogens because their binding affinity for the estrogen receptor is 1,000-fold lower than estradiol. However, if the bioavailability of DES, *o,p'*-DDT, and OP is greater than estradiol in biological systems, the estrogenicity of these compounds relative to estradiol would be enhanced. This enhancement would be further magnified if the compounds were not readily degraded and bioaccumulated in the body or if they persisted in the serum or cytosol.

The observation that alligator serum has a lower binding activity for xenoestrogens when compared to human serum could indicate a species-specific difference in sensitivity. The differences noted between alligator and human sera could be accounted for by differences in plasma pro-

tein composition. Human serum is known to have a specific sex hormone binding protein (20) as does alligator serum (24; Crain and Guillelte, unpublished data). The concentration of the SHBG in alligators is reduced during the breeding season (24), when the serum used was obtained. However, the concentration of SHBG in alligator serum was comparable to that reported for human serum (20,24). As far as we know, only one report exists on the concentration of SHBG in alligators (24), and therefore this area awaits further research. The differences in response could be due to either seasonal variation in serum protein composition or to species-specific differences in serum protein composition rather than to the absence of sex hormone binding proteins in alligators.

It is important to note that the IC₅₀ values for human and alligator sera with 17 β -estradiol were approximately equivalent. This leads us to conclude that the differences in serum binding with xenoestrogens is due to species-specific differences in serum protein composition. Nevertheless, our data suggest that a bioassay could be developed to measure the sensitivity of a species to xenoestrogens by testing a species' serum for binding activity in the YES system. Use of whole serum from various species would provide data on interspecies variation in the potential bioavailability of xenoestrogens. With respect to the alligator, whole serum rather than the individual binding proteins was used because the identification and characterization of binding proteins has been very limited (24). Importantly, it is the binding characteristics of whole serum, not a single plasma protein, that will define the

bioavailability of xenoestrogens. This information, coupled with data on the capacity of the estrogen receptor to recognize specific xenoestrogens, and the concentrations of various xenoestrogens in a species' environment, could be predictive of potential health risks.

The impact of xenoestrogens on the health of humans and wildlife will require identifying the differences between natural estrogens and xenoestrogens. Several distinctions between natural estrogens and xenoestrogens are beginning to emerge. Data to date indicate that xenoestrogens are metabolized at a significantly slower rate than estradiol, suggesting that xenoestrogens may accumulate in lipids or persist in serum to concentrations sufficient to activate the estrogen receptor. In addition, slower metabolism may account for the increased nuclear retention of the estrogen receptor after binding xenoestrogens compared to estradiol. As shown in this study, the bioavailability of xenoestrogens in serum is apparently greater than that of estradiol.

In conclusion, the YES system can be used to identify and characterize xenoestrogens. The xenoestrogens tested have greater bioavailability in serum than estradiol, indicating a fundamental difference between natural estrogens and some xenoestrogens. We hypothesize that increased availability of xenoestrogens in serum would lead to a greater response at the cellular level. The difference in the ability of human and alligator serum to bind xenoestrogens may indicate that a species-specific sensitivity to xenoestrogens exists. In addition, future studies need to be performed on the binding affinity of the estrogen receptor from various species for the xenoestrogens found in their environment. For as we have proposed, the estrogenicity of various xenoestrogens depends on their availability, metabolism, and binding affinity to the estrogen receptor.

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